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Research Article

Biotransformation of brominated flame retardants into potentially endocrine-disrupting metabolites, with special attention to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47)

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In this study, the endocrine-disrupting (ED) potency of metabolites from brominated flame retardants (BFRs) was determined. Metabolites were obtained by incubating single-parent compound BFRs with phenobarbital-induced rat liver microsomes. Incubation extracts were tested in seven *in vitro* bioassays for their potency to compete with thyroxine for binding to transthyretin (TTR), to inhibit estradiol-sulfotransferase (E2SULT), to interact with thyroid hormone-mediated cell proliferation, and to (in-)activate the androgen, progesterone, estrogen, or aryl hydrocarbon receptor. For most BFRs, TTR-binding potencies, and to a lesser extent E2SULT-inhibiting potencies, significantly increased after biotransformation. Microsomal incubation had less pronounced effects on other ED modes of action, due to low biotransformation efficiency and background activities determined in control incubations without BFRs. Moreover, cell-based bioassays suffered from cytotoxicity from metabolites of lower-brominated polybrominated diphenyl ethers. For the environmentally relevant 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), six hydroxylated metabolites were identified. Individual metabolites had TTR-binding and E2SULT-inhibiting potencies 160 – 1600 and 2.2 – 220 times higher than BDE-47 itself, whereas their combined potencies in a realistic mixture were well predicted *via* concentration addition. In combination with other environmentally relevant hydroxylated organohalogens acting on TTR-binding and E2SULT inhibition, internal exposure to BFR metabolites may significantly contribute to the overall risk of endocrine disruption.

Keywords: Endocrine disruption / Hydroxylated polybrominated diphenyl ether / Metabolism / Polybrominated diphenyl ether / Risk assessment

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1 Introduction

Over the last few years, increasing evidence has become available that some brominated flame retardants (BFRs) have endocrine-disrupting (ED) potencies [1 – 3]. In an *in vitro* screening, five different ED profiles of BFRs could be distinguished based on their potency to interfere with thyroidal, estrogenic, androgenic, progestagenic, and aryl

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Abbreviations: 246-TBP, 2,4,6-tribromophenol; **AR**, androgen receptor; **BDE**, brominated diphenyl ether; **BFR**, brominated flame retardant; **CA**, concentration addition; **CALUX**, chemically activated luciferase gene expression; **CYP**, cytochrome P450; **DHT**, dihydrotestosterone; **DIPE**, diisopropyl ether; **DR**, dioxin receptor (or aryl hydrocarbon receptor); **E2**, estradiol; **E2SULT**, estradiolsulfotransferase; **ED**, endocrine disrupting; **EQ**, equivalent; **ER**, estrogen receptor; **HBCD**, hexabromocyclododecane; **IC_{50}**, concentration yielding 50% inhibition; **OH-PBDE**, hydroxylated PBDE; **OH-PCB**, hydroxylated polychlorinated biphenyl; **PBDE**, polybrominated diphenyl ether; **PCB**, polychlorinated biphenyl; **PCP**, pentachlorophenol; **PR**, progesterone receptor; **REP**, relative potency; **T3**, triiodothyronine; **T4**, thyroxine; **TBBPA**, tetrabromobisphenol-A; **TBBPA-DBPE**, tetrabromobisphenol-A dibromopropyl ether; **THF**, tetrahydrofuran; **TM**, technical mixture; **TR**, thyroid receptor; **TTR**, transthyretin

hydrocarbon receptor-mediated pathways [4]. Since many BFRs can be metabolized *in vivo* [5] into potentially ED active compounds, hazard assessment of BFRs requires not only information about the *in vitro* ED potency of the parent compounds but also of their metabolites. For instance, Meerts *et al.* [6] demonstrated that the estrogen receptor (ER)-activating potency of polybrominated diphenyl ethers (PBDEs) increased considerably after cytochrome P-450-mediated biotransformation. Also, Hamers *et al.* [4] demonstrated a completely different ED profile for 2,2′,4,4′-brominated diphenyl ether (BDE-47) compared to its *ortho*-hydroxylated metabolite 6-OH-BDE-47. Two endocrine related modes of action were in particular higher for 6-OH-BDE-47 than for BDE-47, *i.e.*, (i) the capacity to compete with thyroxine (T4; the transport form of thyroid hormone) for binding to human transthyretin (TTR; a T4-transporting protein in plasma), and (ii) the capacity to inhibit estradiol-sulfotransferase (E2SULT), an enzyme responsible for sulfonation and subsequent inactivation of the endogenous hormone estradiol.

The *in vivo* relevance of hydroxylated PBDE-metabolites (OH-PBDEs) has been demonstrated in several experimental studies reporting the occurrence of a series of OH-PBDEs in the blood and feces of PBDE-exposed rat and mice [5, 7]. Almost 20 OH-PBDE-metabolites have been identified in rat blood after exposure to a mixture of tetra- to decaBDEs [9]. In these studies, the majority of OH-PBDEs were substituted with the hydroxyl group in either the *meta*- or *para*-positions. Many of these experimentally identified OH-PBDEs have recently also been identified in human plasma, indicating their tendency to accumulate in the blood compartment [10]. Remarkably, *ortho*-substituted 6-OH-BDE-47 is one of the most abundant OH-PBDEs in human blood samples, but is only a minor metabolite from BDE-47 in the rat [8]. Possibly, the 6-OH-BDE-47 in human blood originates from natural production by algae, mussels, or fish, as has been demonstrated for a series of environmentally occurring *ortho*-hydroxylated PBDEs [11]. It is not yet known to what extent these compounds are making their way into mammals and humans.

In the present study, we studied changes in the ED profile of BFRs after biotransformation. Hydroxylated BFR metabolites were prepared by biotransformation of a single-parent compound BFR in the presence of phenobarbital-induced rat liver microsomes and NADPH. Phenobarbital was chosen as cytochrome P450 (CYP) inducer, since Meerts *et al.* [12] demonstrated that biotransformation with CYP2B-enriched microsomes resulted in PBDE metabolites with higher TTR-binding potency than biotransformation with CYP1A- or CYP4A3-induced microsomes. In addition, recent studies indicated that BFRs themselves are potent CYP2B inducers *in vivo* [13, 14]. Incubation periods were based on biotransformation rates described previously by Harju *et al.* [15]. The aim of this study was threefold, *i.e.,* (i) to determine possible changes in *in vitro* TTR-binding and E2SULT-inhibiting potencies of BFRs after biotransformation, (ii) to determine possible changes in the potencies of BFRs after biotransformation for disruption of five other hormone-regulated modes of action, *i.e.,* cell proliferation mediated by the active form of thyroid hormone (triiodothyronine (T3)) and interaction with the androgen, progesterone, estrogen, and dioxin (or aryl hydrocarbon) receptor (AR, PR, ER, and DR, respectively), and (iii) to identify and quantify the different hydroxylated metabolites in incubation extracts and their contribution to the ED potency of the metabolite mixture formed after incubation. For this last goal, we focused particularly on BDE-47, given its abundant presence in the environment and the availability of standards of its hydroxylated metabolites.

2 Materials and methods

2.1 BFRs and BDE-47 metabolites

Environmentally relevant PBDE congeners number 28, 47, 49, 99, 100, 153, 155, and 181 as well as 6-OH-BDE-47 (>99%; cleaned up on charcoal column to remove possible impurities of brominated dibenzofurans and dioxins) were synthesized as described elsewhere [16, 17]. BDE-209 was purchased from Fluka Chemie and purified on a charcoal column. Less environmentally relevant PBDE congeners 19, 38, 39, 79, 127, and 185 (>98%) were bought from Accu Standard. Tetrabromobisphenol-A (TBBPA; >97%) and 2,4,6-tribromophenol (246-TBP; >98%) were obtained from Aldrich, a technical mixture (TM) of hexabromocyclododecane (HBCD) from Dead Sea Bromine Group, the individual HBCD diastereomers *alpha*, *beta*, and *gamma* (>97%) from Cambridge Isotope Laboratories, and a TM of tetrabromobisphenol A-dibromopropylether (TBBPA-DBPE) from Broomchemie BV Terneuzen. For all compounds, stock solutions were prepared in DMSO [4].

Six mono-hydroxylated tetrabrominated metabolites that were theoretically expected to be formed from BDE-47 by direct hydroxylation of free positions or by a 1,2-bromine shift *via* an arene oxide were synthesized by Marsh and coworkers [18], *i.e.,* 3-OH-2,2′,4,4′-tetraBDE (3-OH-BDE-47), 5-OH-2,2′,4,4′-tetraBDE (5-OH-BDE-47), 6-OH-2,2′,4,4′-tetraBDE (6-OH-BDE-47), 4-OH-2,2′,3,4′-tetraBDE (4-OH-BDE-42), 4′-OH-2,2′,4,5′-tetraBDE (4′-OH-BDE-49), and 2′-OH-2,3′,4,4′-tetraBDE (2′-OH-BDE-66). OH-PBDEs were dissolved in DMSO for bioassay testing or in iso-octane for use as authentic reference standards. In addition, an artificial mixture was reconstituted in DMSO consisting of BDE-47 and its six identified metabolites in exactly the same composition as determined after biotransformation. This mixture was used to determine the combined activity of BDE-47 and its metabolites in the TTR-binding assay and the E2SULT inhibition assay.

2.2 Microsomal incubation of BFRs and extraction

Biotransformation was performed with BFRs, for which Harju *et al.* [15] determined half-lives <700 min (see Table 1). BDE-153 and BDE-209, which have half-lives ⩾700 min were also incubated as negative control substances. Incubation of the individual BFRs and extraction of the incubation mixture were performed as described elsewhere [15], with modified volumes and concentrations to obtain sufficient amounts of biotransformed material for bioassay testing. BFRs were metabolized using rat hepatic microsomes from phenobarbital exposed male Wistar WU rats (0.1% w/v in drinking water for 7 days) [12]. Incubation was performed in 100 mM Tris-HCl (pH 7.8), containing 25 μM parent compound (*i.e.,* 1% v/v dilution of 2.5 mM stock solution in DMSO), 1 mg/mL microsomal protein, and 1 mM NADPH, which was added to start the incubation (final volume of 10 mL). Control incubations received Tris-HCl buffer instead of NADPH. To prevent NADPH depletion from the incubation mixture, an additional 50 μL of 200 mM NADPH in Tris-HCl was added every 15 min of the incubation period. Based on its half-life in earlier experiments [15], a specific incubation period ranging from 15 to 90 min was assigned to each individual BFR (Table 1). Incubation was stopped by denaturation of microsomal proteins with 10 mL of ice-cold methanol. After centrifugation of the incubation mixtures (15 s, 1000 *g*, 4°C), 5 mL of Tris-HCl buffer was added to the collected supernatant and three liquid:liquid extractions were performed by consecutive shaking with 3 × 10 mL diisopropyl ether (DIPE). To facilitate exchange of metabolites to the DIPE phase, 1 mL of 1 M HCl was added to the aqueous phase during the third extraction step. DIPE phases were collected and pooled, evaporated under a gentle nitrogen flow and redissolved in 250 μL DMSO, yielding a stock solution equivalent to 1 mM of parent compound, assuming 100% extraction efficiency.

Metabolite recovery was checked by triplicate control incubations with 6-OH-BDE-47 and microsomes in the absence of NADPH, which were extracted similar to the

Table 1. Characteristics of BFR biotransformation by phenobarbital-induced rat liver microsomes and the TTR-binding potency of parent compounds and metabolites (ND, not determined)

Compound	Substitution pattern	Biotransformation rate ($t_{1/2}$ [min]) in 1-μM mixtures[a)]	Incubation time [min] for biotransformation of individual BFRs in the present study[b]	IC_{50} [μM] in TTR-binding assay[c)]	
				Parent compound[d)]	Biotransformation extract[e)]
BDE-19	2,2′,6	2	15	–	0.1 – 1.0
BDE-28	2,4,4′	25	30	–	0.1 – 1.0
BDE-38	3,4,5	4	15	>25	0.1 – 1.0
BDE-39	3,4′,5	58	90	–	0.1 – 1.0
BDE-47	2,2′,4,4′	95	90	>25	1.0 – 10
BDE-49	2,2′,4,5′	4	15	>25	0.1 – 1.0
BDE-79	3,3′,4,5′	71	90	–	1.0 – 10
BDE-99	2,2′,4,4′,5	170	90	–	1.0 – 10
BDE-100	2,2′,4,4′,6	150	90	–	1.0 – 10
BDE-153	2,2′,4,4′,5,5′	>700	90	–	>10
BDE-155	2,2′,4,4′,6,6′	29	60	–	0.1 – 1.0
BDE-185	2,2′,3,4,5,5′,6	63	90	7.4	1.0 – 10
BDE-209	2,2′3,3′,4,4′,5,5′,6,6′	>700	90	–	–
TBBPA		60	90	0.031	0.1 – 1.0
246-TBP		26	60	0.0048	0.1 – 1.0
6-OH-BDE-47		7	15	0.18	0.1 – 1.0
HBCD TM		ND[f)]	90	–	–
HBCD alpha		170	90	12	–
HBCD beta		7	30	25	–
HBCD gamma		25	90	–	–
TBBPA-DBPE		ND	90	5.2	>10

a) Reported by Harju *et al.* [15]
b) No metabolite mixtures were prepared for BFRs with half-lives >200 min [15], except for BDE-153 and BDE-209, for which microsomal extracts were prepared as for the negative controls
c) Means no response at maximum test concentration
d) Reported by Hamers *et al.* [4]; maximum test concentration = 62.5 μM
e) Concentration expressed as equivalent to the parent compound, assuming 100% extraction recovery; maximum test concentration = 25 μM
f) Harju *et al.* [15] determined biotransformation rates for all individual congeners, and not for the TM that mainly consisted of HBCD gamma

parent compound incubations. Concentrations of 6-OH-BDE-47 were determined in the extracts from this control incubation according to a clean-up method originally developed for analysis of hydroxylated metabolites of polychlorinated biphenyls (OH-PCBs; [19]). Extracts were redissolved in hexane and methylated overnight with ethereal diazomethane at 4°C. Clean-up was performed using a sulfuric acid-silica gel column (1 g of concentrated sulfuric acid:silica gel (1:2 w/w)). The compounds were eluted from the column with hexane:dichloromethane (8:2 v/v). After elution the extracts were quantitatively transferred into autosampler vials for GC-MS analysis.

2.3 Additional preparation and analysis of cleaned BDE-47 metabolites

Using an adjusted protocol, additional incubations were performed with the environmentally relevant BDE-47 to (i) reduce background responses in the E2SULT assay and (ii) to identify which metabolites are responsible for the increased TTR and E2SULT bioassay responses for BDE-47 after biotransformation. According to Van Lipzig *et al.* [20], BDE-47 was incubated similarly as described above, but the reaction was stopped with 1 mL of perchloric acid on ice. Metabolites were liquid–liquid extracted by shaking the incubation mixture for 5 min with 10 mL DIPE, followed by 5 min centrifugation (1000 *g*, 4°C) and collection of the DIPE fraction. After repetition of this extraction step, both DIPE fractions were pooled, evaporated under a gentle nitrogen flow, and redissolved in 100 μL water:ACN (1:1 v/v). An extra clean-up step was included using an HPLC (Varian 9012) with a Waters column (ODS2 5 μm; 4.6 × 150 mm; 0.6 mL/min flow rate). Retention times of background peaks and metabolite peaks were determined in 10-μL aliquot of the extract on a UV-VIS detector (Shimadzu SPD-10Avp; λ = 254 and 290 nm). A solvent gradient was used starting at 70% solvent A (90% water, 10% ACN, 0.1% TFA) and 30% solvent B (10% water, 90% ACN, 0.1% TFA) linearly changing to 60% of solvent B at t = 10 min and 100% at t = 20 min, which was continued until t = 30 min. By comparing chromatograms obtained from BDE-47 incubations and control incubations, metabolites could be distinguished from background peaks in fractions F1 (13.5–19.6 min) and F2 (20.2–23.0 min) of the extract. Subsequently, the remaining 90 μL of the extract was cleaned on the HPLC under the same conditions as described above. Fractions F1 and F2 were collected, pooled, evaporated under a gentle nitrogen flow, and redissolved in 1 mL THF.

Ten percent of this cleaned extract (100 μL) was used for metabolite identification and quantification. After evaporation of THF, the residues were redissolved in 1 mL hexane and derivatized with ethereal diazomethane and cleaned on a sulfuric acid-silica gel column, as described above. The sulfuric acid cleaned extract was redissolved in 100 μL isooctane and analyzed by GC-MS (Agilent 6890 with 5973 MS). According to Marsh *et al.* [8], an SPTM 2331 column (30 m, 0.25 mm, 0.2 μm film thickness; Supelco, Belefonte USA) was used with a column temperature program starting at 80°C (1 min), ramped with 20°C min^{-1} to 200°C (1 min), and with 3°C min^{-1} to 270°C (12 min). The injector (Gerstel CIS4) was operated in splitless mode (80°C, ramped with 12°C s^{-1} to 275°C at 0.06 min) and the transfer line temperature was 280°C. Helium was used as carrier gas. The MS was run in electron impact mode. The electron energy was 70 eV, MS source temperature was 230°C, and the MS QUAD temperature was 150°C. The MS was operating in SIM mode scanning for masses 514, 516, and 518 *m/z*. For identification and quantification, metabolite standards were derivatized and analyzed similarly.

The remaining 90% (=900 μL) of the HPLC-cleaned extract was evaporated and redissolved in 202.5 μL DMSO, yielding a stock solution of metabolites equivalent to 1 mM of parent compound assuming no compound was lost during the clean-up steps described previously.

2.4 *In vitro* bioassays

Extracts from biotransformation incubations were tested in seven different bioassays for 12 different endocrine disrupting modes of actions, exactly according to the methods described for the parent compounds [4]. The capacity of BFR metabolites to compete with T4 for binding to human TTR was investigated in a TTR-binding assay with [^{125}I]-radiolabeled T4 [21] with modifications. The potency of BFR metabolites to inhibit sulfonation of estradiol was determined as a decrease in the formation of estradiol-sulfate after BFR incubation with tritium-labeled estradiol ([^{3}H]E2), recombinant human estradiol sulfotransferase, and the sulfonate donor 3′-phosphoadenosine-5′-phosphosulphate in the E2SULT assay [22]. Interactions of BFR metabolites with the androgen receptor, progesterone receptor, estrogen receptor, and dioxin receptor were tested in the AR-, PR-, ER-, and DR-CALUX® bioassay, respectively [23]. CALUX bioassays (Chemically Activated LUciferase gene eXpression; BioDetection Systems BV (BDS), Amsterdam, The Netherlands) are reporter cell lines carrying a luciferase gene under the transcriptional control of response elements for activated receptors of interest. The potency of BFR metabolites to interact with thyroid receptor (TR) was tested in the T-screen, a functional assay based on the T3-dependent cell proliferation of the rat pituitary tumor cell line GH3 [27]. By testing the biotransformation extracts in the absence and presence of a positive standard compound, both agonistic and antagonistic potencies could be determined in the cell-based CALUX bioassays and T-Screen. Cytotoxicity of biotransformation extracts was tested as a decrease in metabolic capacity to reduce blue resazurin into pink resorufin in a cell viability bioassay [28].

Table 2. Endocrine-disrupting potency of parent compounds (PC; Hamers *et al.* [4]) and their biotransformation extracts (BE; current study) in the cell-based bioassays[a)]

Test compound	ER-CALUX				DR-CALUX				AR-CALUX		PR-CALUX				T-Screen	
	agonism		antagonism		agonism		antagonism		antagonism		antagonism		potentiation		antagonism	
	PC	BE	PC	BE	PC	BE	PC	BE	PC	BE	PC	BE	PC	BE	PC	BE
Control	–	37 – 42%[b)]	–	≈	–	≈	–	29-39[b)]	–	≈	–	≈	–	≈	–	≈
BDE-19	M	≈	–	≈	–	≈	–	↑[c)]	VH	↓[d)]	H	↓	M	ct	–	ct
BDE-28	L	≈	–	≈	–	≈	M	ct	M	≈	L	≈	H	ct	–	ct
BDE-38	M	≈	–	≈	H	ct	–	ct	M	≈	L	≈	L	ct	–	ct
BDE-39	–	≈	–	≈	–	≈	L	≈	M	≈	L	≈	–	ct	–	ct
BDE-47	L	≈	–	≈	–	≈	M	≈	M	≈	L	≈	–	ct	–	ct
BDE-49	M	≈	–	≈	L	ct	L	ct	H	≈	M	≈	VH	ct	–	ct
BDE-79	L	≈	L	↓	M	↓	–	↑[c)]	M	≈	L	≈	–	≈	–	≈
BDE-99	–	≈	–	≈	L	↓	L	↓	M	≈	L	≈	–	≈	–	≈
BDE-100	M	≈	–	≈	–	≈	L	↓	VH	≈	M	≈	H	↓	–	≈
BDE-153	–	≈	–	≈	H	↓	-	≈	L	≈	M	≈	–	≈	–	≈
BDE-155	M	≈	–	≈	–	–	–	≈	M	≈	M	≈	M	≈	–	≈
BDE-185	–	↑[d)]	L	↓	–	≈	–	≈	L	≈	M	≈	–	↑[d)]	–	≈
BDE-209	–	≈	–	≈	–	≈	–	≈	–	≈	–	≈	–	≈	–	≈
TBBPA	–	≈	–	≈	–	≈	–	≈	–	≈	–	≈	M	–	–	≈
246-TBP	–	≈	M	↓	–	≈	–	≈	L	≈	L	≈	–	≈	–	≈
6-OH-BDE-47	–	≈	H	↓	M	ct	–	ct	M	≈	M	≈	–	≈	–	≈
HBCD TM	–	≈	L	↓	–	≈	L	↓	L	≈	M	≈	M	≈	–	≈
HBCD alpha	–	≈	–	≈	–	≈	M	↓	M	≈	M	≈	H	≈	–	≈
HBCD beta	–	≈	L	↓	–	≈	L	≈	L	≈	L	≈	M	≈	–	≈
HBCD gamma	–	≈	M	↓	–	≈	L	↓	M	≈	M	≈	H	≈	–	≈
TBBPA-DBPE	–	≈	–	≈	–	≈	–	≈	–	↑[d)]	–	≈	–	–	–	≈

a) No potency; L: low potency; M: moderate potency; H: high potency; VH: very high potency; ≈: similar potency after biotransformation compared with parent compound; ↑: increased potency after biotransformation compared with parent compound; ↓: decreased potency after biotransformation compared with parent compound; ct: cytotoxic

b) Control incubation extracts show ER-agonistic and DR-antagonistic potencies of 37 – 42% and 29 – 39% of the maximum response, respectively

c) Cytotoxicity was not observed in the cell viability assay, but cannot be excluded

d) Results are illustrated in Fig. 3

2.5 Experimental set-up and data analysis

Biotransformation extracts from all 21 selected BFRs was tested in all selected bioassays in four different tenfold dilutions. Assuming 100% extraction recovery, maximum test concentrations (expressed as equivalent to the parent compound) were 25 μM (TTR-binding assay), 10 μM (E2SULT-assay), 4 μM (DR-CALUX bioassay), and 1 μM (ER-, AR-, and PR-CALUX bioassays). For the TTR-binding assay ($n = 2$), IC_{50} values were assessed for the biotransformation extracts assuming 100% extraction recovery. These results should be regarded semi-quantitatively, given the unknown extraction efficiencies of the individual metabolites produced *per* parent compound. Results from the E2SULT bioassay and the cell-based T-Screen and CALUX bioassays ($n = 1$) could only be treated qualitatively, since quantification was not only hampered by unknown extraction efficiencies but also by background ED activity, low biotransformation efficiencies, and/or cytotoxicity (see Section 3). For ED-inactive parent compounds [4], changes in ED potency were only reported (Table 2) if the highest test concentration of the biotransformation extracts showed a response greater than 25% of the maximum response found for a positive standard. For ED active parent compounds [4], changes were only reported (Table 2) if the ED potency in the highest test concentration of the biotransformation extracts either had completely disappeared (in most cases) or differed more than 25% in response compared to the parent compound.

Additional experiments were performed to determine the TTR-binding and E2SULT-inhibiting potencies of the individual parent compound BDE-47 and its six hydroxylated metabolites. The combined TTR-binding and E2SULT-inhibiting potencies of BDE-47 and its metabolites were determined in a cleaned biotransformation extract of BDE-47 and in a reconstituted artificial mixture reflecting the exact ratio between parent compound and metabolites as determined in the biotransformation extract. Experiments were performed in duplicate and dose-response curves were calculated using a sigmoid curve-fitting model with Hill slope. Relative potency (REP) factors were determined for BDE-47 and its metabolites by comparing their IC_{50} value with the IC_{50} value of reference materials T4 (TTR-binding

assay) or pentachlorophenol (PCP; E2SULT assay). Using these REP factors, concentrations of biotransformation extracts and reconstituted mixtures could be expressed in terms of T4-equivalent (T4-EQ) or PCP-equivalent (PCP-EQ) concentrations. Significant differences between dose-response curves of reference materials, biotransformation extracts, and reconstituted mixtures were determined for each individual experiment with a Student's *t*-test on slope estimates and IC_{50} estimates.

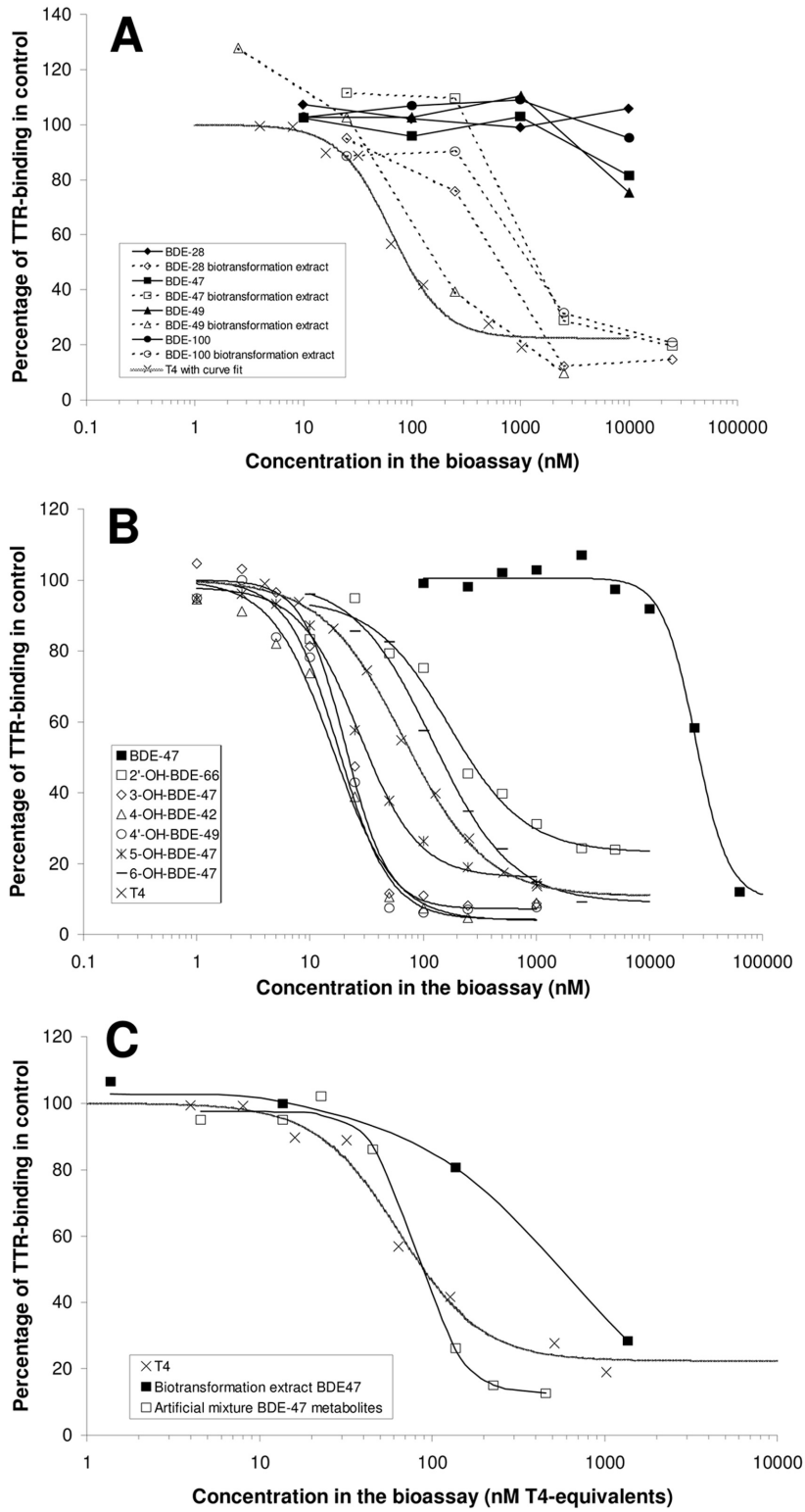


Figure 1. Dose-response curves for the TTR-binding potencies of parent PBDE compounds and PBDE metabolites compared to the natural ligand T4. (A) PBDEs before and after biotransformation by hepatic microsomes from phenobarbital induced rats. (B) BDE-47 and all its six identified mono-hydroxylated metabolites. (C) The biotransformation extract of BDE-47 and an artificial mixture composed of BDE-47 and its six identified mono-hydroxylated metabolites in exactly the same proportions as found in the biotransformation extract.

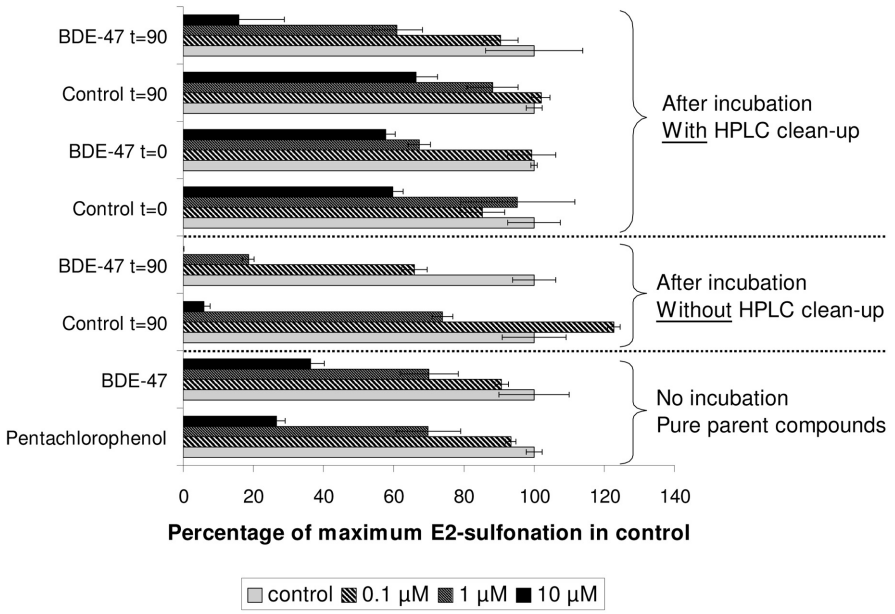


Figure 2. Inhibition of estradiol-sulfotransferase (E2SULT; mean ± SD) by parent compounds pentachlorophenol (positive control) and BDE-47 compared to inhibition by the extracts from incubation mixtures with DMSO (control) or BDE-47. Without clean-up, the increase in the E2SULT-inhibiting potency of the BDE-47 after biotransformation could hardly be distinguished from the background inhibition by the control extracts. Although HPLC clean-up improved the resolution between BDE-47 metabolites and control incubations, background inhibition was too large to allow further quantification for the BDE-47 metabolites.

3 Results

3.1 Screening for *in vitro* ED potencies of BFR metabolites

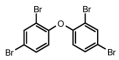
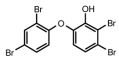
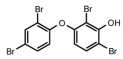
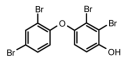
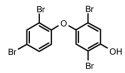
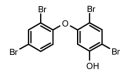
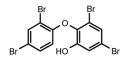
The most pronounced differences between parent compounds and biotransformation extracts were found for the T4-replacing potency of PBDEs in the TTR-binding assay (Table 1). At concentrations where parent PBDE compounds had no or low T4-competitive potency, clear dose-response relationships were obtained for PBDE metabolites (Fig. 1a). In fact, the increase in potency is probably even larger than shown in Fig. 1a, since the recovery experiment yielded an extraction efficiency of 65.1 ± 2.3% (mean ± SD) for 6-OH-BDE-47, suggesting that the assumed value of 100% overestimated the actual recovery of hydroxylated PBDE metabolites. Although TTR-binding potencies of TBBPA and 246-TBP were still relatively high after biotransformation, they were lower than found for the original very potent parent compounds (Table 1).

For most PBDEs, the E2SULT-inhibiting potency also increased after biotransformation, but the E2SULT-inhibiting potency of the extracts from microsomal control incubations (*i.e.,* without BFR added) was too high to make direct comparisons between E2SULT-inhibiting potencies of individual parent compounds and their corresponding biotransformation extracts. For example, 10 µM of BDE-47 caused 64% of E2SULT inhibition, whereas the corresponding concentration of its biotransformation extract caused 100% inhibition (Fig. 2). This change in potencies, however, could not be attributed to the presence of BDE-47 metabolites, since control extracts containing no metabolites caused 94% of E2SULT inhibition at corresponding concentrations. To decrease background activity from microsomal incubations, additional HPLC clean-up experiments were performed as described in Section 3.2.

In the cell-based bioassays, biotransformation extracts of the lower-brominated tri- and tetra-BDEs caused cytotoxicity at concentrations equivalent to those where parent compounds caused a response (Table 2). During visual inspection of the cells, cytotoxicity was observed in the rat GH3 pituitary tumor cells of the T-Screen bioassay, and the rat H4IIE hepato-carcinoma cells of the DR-CALUX bioassay, but not in the human T47D breast cancer cells of the ER-CALUX bioassay and the human U2OS osteoblast cells of the AR- and PR-CALUX bioassays. Visually observed cytotoxicity was confirmed by a decreased metabolization of resazurin in the cell viability bioassay (data not shown).

For the ER-antagonistic parent compounds BDE-185, 246-TBP, 6-OH-BDE-47, HBCD TM, HBCD beta, and HBCD gamma [4], a decrease or complete absence of anti-estrogenic potency was found after biotransformation (Table 2). Similarly, the dioxin-like potency of DR-agonistic parent compounds BDE-79, BDE-99, BDE-153, and 6-OH-BDE-47 [4] decreased or disappeared after biotransformation (Table 2). These decreases in ER-antagonistic and DR-agonistic potency may be attributed to biotransformation of the active parent compound, but a (partial) counterbalance by unknown ER-agonistic and DR-antagonistic

Table 3. Characteristics of BDE-47 and its six hydroxylated metabolites identified after incubations with phenobarbital-induced rat microsomes

Characteristic	BDE-47	2′-OH-BDE-66	3-OH-BDE-47	4-OH-BDE-42	4′-OH-BDE49	5-OH-BDE-47	6-OH-BDE-47
Molecular structure							
Measured contribution to the molar composition of the whole extract including the parent compound BDE-47(%)	98	0.30	0.84	0.10	0.54	0.074	0.022
Measured contribution to the molar composition of the whole extract excluding the parent compound BDE-47 (%)	Not applicable	16	45	5.2	29	3.9	1.2
TTR-binding assay							
IC_{50} (nM)[a)]	36×10^3	17×10^1	17	19	19	25	15×10^1
Relative Potency towards T4 (T4-REP)[b)]	0.0025	0.65	4.0	3.5	3.5	3.0	0.39
Calculated contribution to TTR-binding potency of the extract (%)	3.9	3.1	54	5.5	30	3.5	0.14
E2SULT inhibition assay							
IC_{50} (nM)[c)]	4.0×10^3	1.8×10^3	23	23	18	1.1×10^2	4.0×10^2
Relative Potency towards pentachlorophenol (PCP-REP)[b)]	0.038	0.083	6.5	6.5	8.3	1.4	0.38
Calculated contribution to E2SULT inhibiting potency of the extract (%)	28	0.16	37	3.4	30	0.59	0.038

a) IC_{50} values for TTR-binding assays are calculated as the point of inflection in a full dose-response curve
b) Relative potencies are determined as the ratio of IC_{50} (reference compound T4 or PCP):IC_{50} (test compound) within each individual experiment
c) IC_{50} values for E2SULT assays are calculated by linear interpolation between two responses located around the 50% inhibition level

compounds in the microsomal extracts cannot be excluded. At the highest test concentration, control extracts from microsomal incubations had ER-agonistic and DR-antagonistic background activities of 37–43% and 29–39%, respectively, of the maximum response (Table 2). The DR-antagonistic background response may explain why the DR-agonistic potency of BDE-153 decreased after biotransformation, whereas this compound is hardly metabolized by phenobarbital-induced rat liver microsomes [15]. On the other hand, DR-antagonistic BDE-99, BDE-100, and HBCD [4] showed a decreased or complete absence of potency after biotransformation, despite the DR-antagonistic background potency.

Although cytotoxicity and background responses hampered interpretation in many cases, some differences were still obvious when comparing the endocrine-disrupting potencies of metabolite extracts with parent compound BFRs in cell-based bioassays.

Only the biotransformation extract of BDE-185 exceeded the background ER-agonistic activity of the control incubations, whereas no ER activation was found for the parent compound (Fig. 3a) [4].

After biotransformation, TBBPA-DBPE became a more potent AR antagonist, whereas the extremely potent antagonists BDE-19 became somewhat less potent (Fig. 3b). For all other compounds, no differences were found between parent compounds and metabolites in AR- and PR-CALUX bioassays (Table 2).

In the presence of T3 (0.25 nM), but not in its absence, biotransformation extracts of BDE-185 (Fig. 3c), TBBPA, and to a lesser extent TBBPA-DBPE enhanced GH3 cell proliferation in the T-Screen. Parent compounds of these BFRs were unable to stimulate GH3 cell proliferation, neither with nor without T3 [4]. Interestingly, the biotransformation extract of BDE-185 tested in combination with 25 nM T3 yielded a T-Screen response 47% higher than the maximum response found for 1000 nM T3 (Fig. 3c). For BDE-100, the T3-enhancing potency reported for the parent compound [4] decreased after biotransformation (data not shown).

3.2 Reducing background response in the E2SULT inhibition assay

Although the E2SULT activity of control extracts improved considerably after HPLC clean-up, the background E2SULT-inhibiting potency could not be completely removed (Fig. 2). At the highest test concentration of the control extract (comparable to the concentration needed to get a 10-μM test concentration), E2SULT activity increased

by a factor of 10 from 6% for the raw extract to 63% for the cleaned extract (Fig. 2), implying a remaining E2SULT-inhibiting potency for the HPLC cleaned incubation extracts of 37%. The E2SULT activity of the BDE-47 biotransformation extracts increased much less after clean-up, *i.e.,* from 0 to 16% (Fig. 2), indicating that the BDE-47 bio-

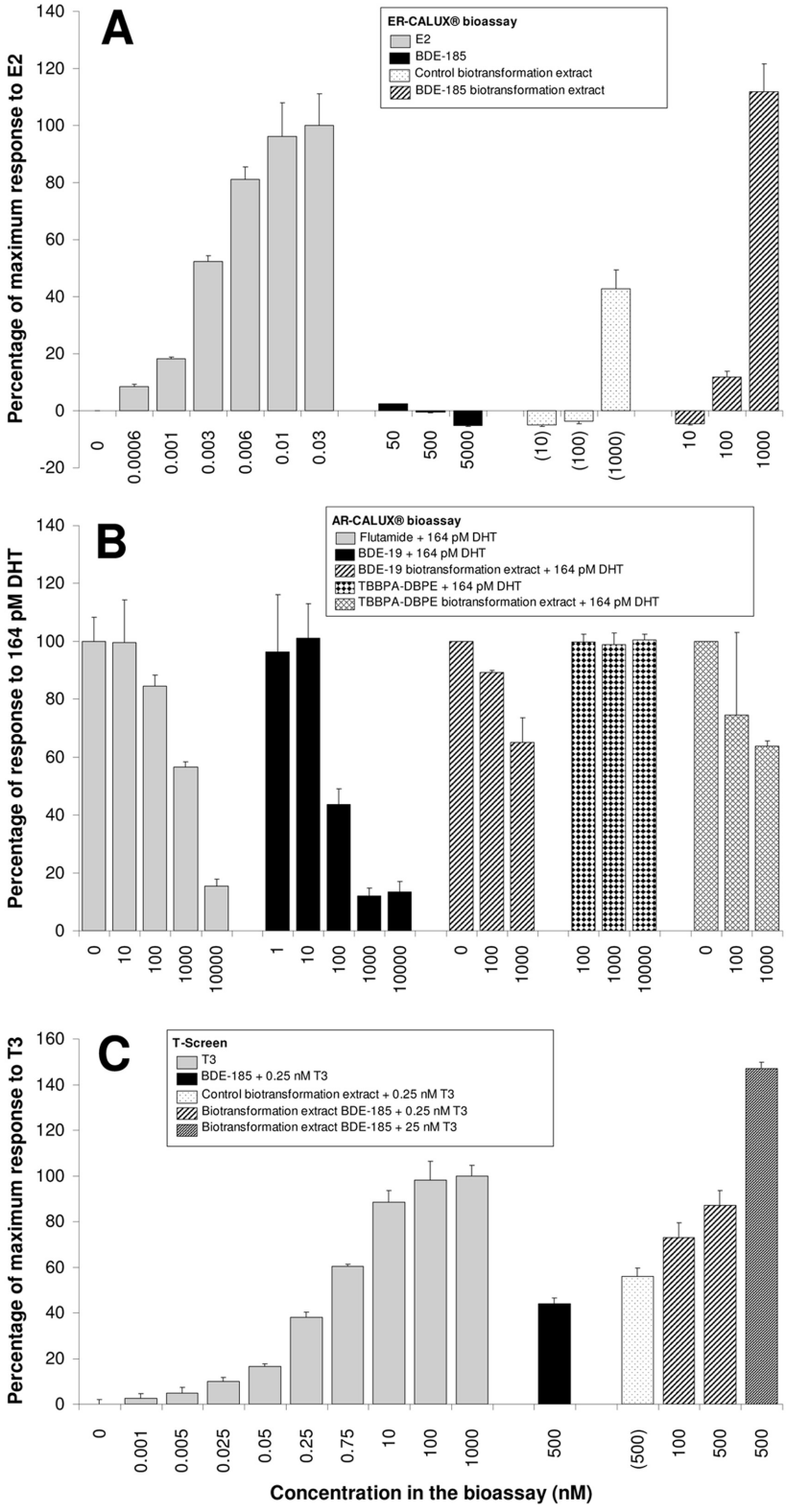


Figure 3. Endocrine-disrupting potencies (mean ± SD) of BFR parent compounds, their corresponding extracts after microsomal incubation with phenobarbital-induced rat liver microsomes, and relevant reference compounds (in gray). Concentrations of control biotransformation extracts (in parentheses) refer to the equivalent concentration of the BFR biotransformation extract when tested in the same dilution. (A) ER activation in ER-CALUX® bioassay; (B) AR inactivation in dihydrotestosterone (DHT)-activated AR-CALUX® bioassay; (C) enhancement of T3-induced cell proliferation in the T-Screen bioassay.

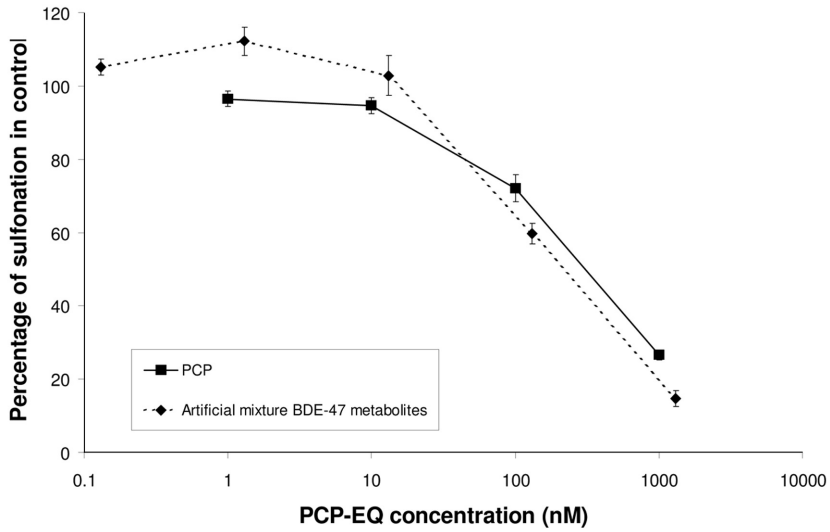


Figure 4. E2SULT-inhibiting potency (mean ± SD) of an artificial mixture of all six identified metabolites together with the parent compound BDE-47 in exactly the same proportions as found in the biotransformation extract. Concentrations are expressed in nM PCP-EQ.

transformation extract contained E2SULT-inhibiting compounds other than the unknown compounds responsible for the background potency.

3.3 TTR-binding and E2SULT-inhibiting potencies of BDE-47 metabolites

All six tentatively expected mono-hydroxy metabolites of BDE-47 were identified in the extracts from the additional BDE-47 incubations (see Section 2.3), *i.e.,* in order of decreasing concentration 3-OH-BDE-47 > 4′-OH-BDE-49 > 2′-OH-BDE-66 ≫ 4-OH-BDE-42 > 5-OH-BDE-47 > 6-OH-BDE-47 (Table 3). The molar quantities of metabolites extracted from the incubations accounted for only 1.6% of the original amount of BDE-47 added, suggesting that more than 98% of the parent compound had not been metabolized. Still, the TTR-binding potency of BDE-47 increased with more than a factor of 10 after incubation (Table 1), suggesting that the metabolites had much higher potency than the parent compound. Indeed, metabolites had TTR-binding potencies 160 – 1600 times higher than the parent compound, when tested separately in the TTR bioassay (Fig. 1b; Table 3). In fact, all four metabolites that were hydroxylated in the *meta*- or *para*-position (*i.e.,* 3-OH-BDE-47, 4-OH-BDE-42, 4′-OH-BDE-49, and 5-OH-BDE-47) were more potent than the natural ligand T4, with relative potencies toward T4 (T4-REP factors) in the range 3 – 4. Based on the analyzed concentrations of the different metabolites and their respective T4-REP factors, the TTR-binding potencies of the test concentrations were expressed in terms of T4-EQ concentrations. To confirm that the assumption of concentration addition (CA) underlying this T4-EQ calculation was correct, an artificial mixture was compiled, consisting of BDE-47 and its six identified metabolites in exactly the same composition as the biotransformation extract (Table 3). Testing this mixture in the TTR-binding assay yielded a dose-response curve that overlapped the dose-response curve for T4 (Fig. 1c), as was to be expected, since concentrations of both curves were expressed in the same unit (*i.e.,* nM T4-EQ). The TTR-binding dose-response curve of the biotransformation extract had significantly shifted to the right side of the T4-curve (Fig. 1c), indicating that T4-EQ concentrations were overestimated and/or the TTR-binding potency of the extract was underestimated.

In principle, a similar approach could be chosen for the E2SULT-inhibiting potency of the incubation extract, but a quantitative comparison between E2SULT-inhibiting potencies of the parent compound BDE-47 and its biotransformation extract was hampered by the relatively high 37% inhibiting potency of control incubations after clean-up (Section 3.2; Fig. 2). For the artificial mixture containing BDE-47 and its six identified metabolites, E2SULT-inhibiting potency could be measured in the bioassay and predicted using pentachlorophenol-EQ (PCP-EQ) concentrations. As in the TTR-binding assay, the dose-response curve of the artificial mixture overlapped with the PCP curve (Fig. 4), confirming that the CA concept was valid for the E2SULT-inhibition assay.

4 Discussion

4.1 Screening the ED potency of BFRs after biotransformation in a battery of bioassays

For many PBDEs, metabolites that were formed during microsomal incubation had higher TTR-binding and E2SULT-inhibiting potencies than the parent compounds (Table 1). In contrast, decreased potencies were found for TBBPA and 246-TBP after biotransformation, indicating that these BFRs with the highest TTR-binding and E2SULT-inhibiting potencies as parent compound [4] were

metabolized into less potent metabolites. Although changes in ED potency after BFR biotransformation were most obvious in the TTR and E2SULT bioassays, results from other bioassays screening for other endocrine-related modes of action also indicated changes in *in vitro* potencies of BFRs after biotransformation. As for the TTR and E2SULT bioassays, differences between bioassay responses to parent compounds and to incubation extracts were in general more pronounced for easily metabolized lower-brominated PBDEs than for less-easily metabolized higher-brominated PBDEs. Higher-brominated PBDEs probably reached an insufficient degree of biotransformation during the incubation periods of 15 – 90 min, despite the fact that these incubation times were sufficient to reach 50 – 80% degradation of parent compounds in an earlier study [15] using the same protocol. To obtain sufficient amounts of metabolites for testing in all bioassays (*i.e.,* 250 μL stock solutions in DMSO analogous to 1 mM of parent compound), however, one adjustment was made to the original protocol [15], *i.e.,* BFR concentrations during incubation were increased from the usual 1 μM to 25 μM. For higher-brominated BFRs, this increase in concentration may have led to low biotransformation efficiencies, yielding biotransformation extracts that were dominated by parent compounds rather than metabolites. Consequently, bioassay responses to metabolite extracts could not be distinguished from responses to parent compounds. This relative decrease in biotransformation efficiency is also clear from the additional incubation with BDE-47, where only 1.6% of the parent compound was metabolized after 90 min (Table 3), compared to 60% in the previous study [15].

Apart from low biotransformation efficiencies, other factors hampered the interpretation of the bioassay screening of the ED potency of BFRs after biotransformation. Cytotoxicity seriously disturbed the applicability of cell-based T-Screen and DR-CALUX bioassays, especially for biotransformation extracts of lower-brominated PBDEs. The exact mechanism behind this cytotoxicity is not known and is currently under investigation in our laboratory [29]. In addition, extracts from microsomal control incubations induced ER-agonistic, DR-antagonistic, T3-enhancing, and E2SULT-inhibiting potencies in the bioassays. These background ED activities of the microsomal extracts may be caused by natural hormones or other endocrine active compounds present in the liver microsomes. Clean-up of the extracts may be a solution for reduction of background activity but only when the hydroxylated nature of the metabolites remains unchanged. Therefore, the most common clean-up method to determine metabolites analytically (*i.e.,* sulfuric acid treatment) is inappropriate, because it involves a methylation of the hydroxy group upon which the bioactivity of the metabolites is lost. Application of a mild chromatographic HPLC clean-up led to a reduction in background E2SULT-inhibiting potency at the highest concentration, but not to a complete elimination (Fig. 2). At the highest test concentration, HPLC cleaned extracts from control incubations inhibited E2SULT activity by 37%, probably due to unidentified compounds found in the HPLC chromatograms (fractions F1 and F2) of extracts from control and BFR incubations (data not shown). Given this relatively large background activity, results from E2SULT experiments with biotransformation extracts were not quantified but only considered qualitatively.

Despite the limitations caused by low biotransformation efficiency, cytotoxicity, and background ED activity of the microsomal incubation extract, a few significant differences in ED potency of BFRs before and after biotransformation could be distinguished in the cell-based bioassays. After biotransformation, a much lower AR-antagonistic potency was found for BDE-19 than for BDE-100, whereas the parent compounds of both BFRs had similar potency (Fig. 3b) [4]. BDE-19 was probably converted much easier by CYP2B-induced rat microsomes into less-potent metabolites than BDE-100. Compared with its parent compound, the biotransformation extract of TBBPA-DBPE had an increased AR-antagonistic potency (Fig. 3b), an increased potency to enhance T3-mediated effects in the T-Screen bioassay (Table 2), and a decreased potency to compete with T4 for TTR-binding (Table 1). Since the TBBPA-DBPE batch used in the present study was a TM rather than a pure parent compound, it cannot be excluded that impurities and their metabolites contributed to this shift in ED potencies. The ED profile of BDE-185 also changed after biotransformation, given the increase in ER-agonistic potency (Fig. 3a) and especially the increased potency to enhance the T3-mediated effects (Fig. 3c). BDE-185 is the only higher-brominated PBDE (>4 bromine atoms) tested for which ED potency other than TTR binding changed after biotransformation. BDE-185 is also the only higher-brominated parent PBDE with two adjacent H-atoms in *meta*- and *para*-positions, which facilitates biotransformation (Table 1) [15].

The fact that the biotransformation extract of BDE-185 tested in combination with 250 nM T3 resulted in a T-Screen response 47% higher than the maximum response found for 1000 nM T3 (Fig. 3c) indicates that BDE-185 metabolites have a synergistic effect on T3-mediated cell proliferation. This synergism may be explained by a different mode of action for BDE-185 metabolites than direct activation of the TH receptor. Possibly, BDE-185 metabolites facilitate T3 transport into the cells *via* induction of transporter proteins such as organic anion transporter peptides (OATPs) [30].

4.2 Profiles of OH-PBDEs determined after *in vitro* and *in vivo* biotransformation

The *in vitro* metabolite profile of BDE-47 obtained after incubation with hepatic microsomes from phenobarbital-induced rats (Table 3) corresponds with the metabolite pro-

file determined in livers from rats orally administered with BDE-47. GC-MS chromatograms for these livers showed three peaks for OH-tetraBDEs [7]. In a later study [8], these peaks were attributed to 4-OH-BDE-42, 4′-OH-BDE-49, and 3-OH-BDE-47 and/or 2′-OH-BDE-66, which were the dominating and most potent TTR-binding metabolites found in the incubation extract (Table 3). In the original study [7], the latter two compounds could not be distinguished due to co-elution on the non-polar column used [8]. Our results, however, suggest that 3-OH-BDE-47 contributes more to this peak than 2′-OH-BDE-66 (Table 3). In feces from the same rats, all six tetrabrominated metabolites were identified, but the metabolite profile was dominated by the *para*-hydroxylated 4′-OH-BDE-49 and to a lesser extent by 4-OH-BDE-42 and 3-OH-BDE-47 (and/or 2′-OH-BDE-66) [8].

As OH-PBDEs are selectively retained by TTR in the blood, plasma concentrations of BDE-47 metabolites directly depend on their TTR-binding potencies. In plasma from rats orally exposed to BDE-47, only two metabolite peaks were found [7], *i.e.,* 4′-OH-BDE-49 and 3-OH-BDE-47/2′-OH-BDE-66 [8]. Remarkably, 4-OH-BDE-42 (*i.e.,* the other metabolite dominating in liver [8] and feces [7]) could not be demonstrated in rat plasma [7], despite its equipotent binding affinities toward human TTR as 4′-OH-BDE-49 and 3-OH-BDE-47 (Table 3). In plasma from rats intraperitoneally dosed with a PBDE mixture including BDE-47, 4-OH-BDE-42 was indeed found, albeit at lower concentrations than 4′-OH-BDE-49 and 3-OH-BDE-47 [9].

All three metabolites were also found in serum from children working (and living) at a dumpsite in Nicaragua, with average concentrations (ng/g lipid) of 9.4 (4-OH-BDE-49), 4.9 (4′-OH-BDE-42), and 3.3 (3-OH-BDE-47) [10]. These human serum samples further contained 6-OH-BDE-47 and 4′-OH-BDE-17 at comparable concentrations of 6.1 and 7.2 ng/g lipid, respectively. 6-OH-BDE-47 contains a hydroxyl-group in an *ortho*-position and bromine groups in 2,4-positions in the non-hydroxylated ring. This structural characteristic (also shared by 2′-OH-BDE-66) is typical for OH-PBDEs that are naturally produced by sponges, tunicates, and/or red algae [8], suggesting that human intake of these compounds can be attributed to consumption of fish or shell-fish exposed to these naturally produced OH-PBDEs. On the other hand, both *ortho*-hydroxylated metabolites were also identified in fish dietary exposed to BDE-47 [30]. Given the similarities in 6-OH-BDE-47 levels in fish-eating and non-fish-eating control groups in the Nicaragua study [10], it is more likely that the relatively high 6-OH-BDE-47 levels in human serum samples are due to metabolization of BDE-47 in man rather than to dietary exposure *via* fish consumption.

Species differences may explain the differences in the contribution of 6-OH-BDE-47 to the BDE-47 metabolite profile observed in human [10] and rat blood [7]. A similar high contribution of 6-OH-BDE-47 to the serum metabolite profile as determined in humans was also found in mice orally exposed to BDE-47 [7]. 4′-OH-BDE-17, the other OH-PBDE found at relatively high concentrations in human blood [10], is a 2,2′,4-tribrominated diphenyl ether, that was also demonstrated in feces from rats orally exposed to BDE-47 [8]. In addition, two other tribrominated hydroxylated metabolites of BDE-47 were found in these rat feces samples, *i.e.,* hydroxylated 2,2′,4-tribromodiphenyl ether (BDE-28) with a 2′OH-(*ortho*) or a 3′OH-(*meta*) group. Tribrominated metabolites were not determined in the present study because no standards were available at the time.

4.3 TTR-binding and E2SULT-inhibiting potency of BDE-47 metabolites

The TTR-binding potency of the artificial mixture, which had the same molar composition as the incubation extract, was well predicted by the CA concept (Fig. 1c). The average IC_{50} ratio mixture:T4 (both expressed in terms of T4-EQ) was 0.98 ± 0.42 (mean ± SD), which was close to the theoretically expected value of 1. For the incubation extract itself, however, an IC_{50} (in terms of T4-EQ) was found that was three to five times higher than for T4. This shift can be caused by an overestimation of the T4-EQ concentration determined according to the principle of CA (x-axis in Fig. 1c) or by an underestimation of the TTR-binding potency determined experimentally in the assay (y-axis in Fig. 1c). An overestimation of the T4-EQ concentration is unlikely, because the calculation was based on actual concentrations in the extract (excluding recovery problems), whereas results with the artificial mixture showed that CA leads to correct T4-EQ calculations. It is more likely that an experimental artifact led to an underestimation of the TTR-binding potency of the incubation extract. Although the extract was cleaned on HPLC, it still contained visible fat residues, which may have caused a decreased availability of the OH-PBDEs for TTR binding in the assay. In addition, the Sephadex columns used to separate free T4 from TTR-bound T4 do not retain all fat, given the observation that eluates were yellow. Possibly, the eluted fat may have acted as a carrier for free T4 through the column, causing leakage of free T4 to the eluate, leading to an overestimation of the TTR-bound T4 and a consequent underestimation of the TTR-binding capacity of the incubation extract.

Using the CA concept, the OH-PBDE levels determined in serum from children working and living on Nicaraguan dumpsites can also be recalculated into T4-EQ concentrations. By summation of the concentrations [10] multiplied with their respective T4-REP factors (Table 3), the T4-EQ concentration of OH-PBDEs in blood plasma was estimated to be 0.102 μg T4-EQ/g lipid or 0.55 nM, assuming a lipid percentage of 0.41% [10] and a density of 1025 g/L (http://hypertextbook.com/facts/2004/MichaelShmukler.shtml) for blood plasma. In a recent risk assessment of prenatal exposure to halogenated phenolic compounds [32], a “no

observed adverse effect level" (NOAEL) was determined for developmental effects such as prolonged estrous cycle and changes in spatial learning behavior in rats. This NOAEL corresponded with estimated levels of 222 μg T4-EQ/g lipid in plasma from rat offspring at post-natal day 4. Comparing this NOAEL with the 0.102 μg T4-EQ/g lipid found for OH-PBDEs in serum from children at Nicaraguan dumpsites, suggests a margin of safety by a factor 2000, which should be sufficient to cover uncertainties to extrapolate from rats to humans. As an alternative, the T4-EQ concentration of OH-PBDEs in blood plasma can be compared directly with the natural T4 concentration in human blood. The 0.55-nM T4-EQ concentration of OH-PBDEs is more than 100 times lower than (*i*) the lower range of total T4 (TT4) in healthy adults estimated to be 64 nM (http://www.thyroidmanager.org/Chapter6/6-frame.htm) and (*ii*) the average IC_{50} value of 70 nM determined for T4 in the present study.

Despite the relatively large margin of safety, children working and living on dumpsites are exposed to potent TTR-binding hydroxylated organohalogens other than BDE-47 metabolites, such as pentachlorophenol, OH-PCBs [33], and possibly TBBPA (not determined), all with T4-REP factors greater than 1 [4, 12, 21, 32]. In addition, it should be realized that vulnerable groups such as children with low thyroid hormone levels or developing fetuses from pregnant women working at the dumpsite might be more at risk.

Using the CA concept, a total E2SULT-inhibiting potency of 1.1 nM or 71 ng PCP-EQ *per* gram of lipid was calculated for the BDE-47 metabolites in serum from children in the Nicaraguan case. Compared to the actual PCP-level of 17 nM or 1200 ng/g lipid determined in these children [33], the contribution of OH-PBDEs to the E2SULT-inhibiting potency is 7% of the contribution of PCP itself. Also, the 1.1 nM PCP-EQ concentration estimated for OH-PBDEs is more than 250 times lower than the average IC_{50} value of 284 nM determined for PCP in the present study. It is worth noting that the 17-nM serum concentration of PCP is only 17 times lower than its *in vitro* IC_{50} value.

4.4 Concluding remarks

The study described in this paper demonstrates that biotransformation can change the *in vitro* ED profile of BFRs. Incubation of parent compounds with microsomes and subsequent extraction of the incubation mixture is an easy method to obtain biologically relevant metabolites. Application of *in vitro* bioassays for screening the ED potency of these extracts, however, was hampered by low biotransformation efficiencies and background ED activities of the microsomal incubation extract. In addition, the applicability of cell-based bioassays was seriously disturbed by cytotoxicity of metabolites from lower-brominated PBDEs.

The most pronounced differences between parent compounds and biotransformation extracts were found for the TTR-binding potency of PBDEs, and to a lesser extent for their E2SULT-inhibiting potency. As for PCBs [21][22], these ED potencies increased after introduction of an OH-group. For BDE-47, six different OH metabolites could be identified, each with TTR-binding and E2SULT-inhibiting potencies 160 – 1600 and 2.2 – 220 times higher, respectively, than the parent compound. Most potent metabolites, *i.e.,* hydroxylated in the *meta*- or *para*-position, were also formed in the highest quantities. Experiments with artificial mixtures of BDE-47 metabolites demonstrated that the TTR-binding and E2SULT-inhibiting potencies could be well predicted applying the CA concept. Using this concept, concentrations of individual BDE-47 metabolites determined in human blood [10] could be aggregated into TTR-binding and E2SULT-inhibiting potencies. The margins of safety between exposure levels (expressed as T4-EQ or PCP-EQ) and *in vivo* effect levels (*i.e.,* more than two orders of magnitude) suggest that BDE-47 metabolites alone pose little risk to humans. It should be realized, however, that environmental exposure to TTR-binding or E2SULT-inhibiting compounds is not restricted to BDE-47 and its metabolites. Humans and wildlife are exposed to complex mixtures containing many other hydroxylated organohalogens, either by direct exposure to parent compounds such as PCP and TBBPA or by indirect exposure to hydroxylated metabolites from PCBs and PBDEs. Since these compounds act on common targets, they have a combined ED potency exceeding the individual potencies of the single constituents of the mixture. Although *in vivo* single-compound toxicity studies traditionally used in hazard assessment of chemical substances do reflect the formation of bioactive metabolites, they do not account for the combined hazard with other compounds and their bioactive metabolites acting on the same target. Therefore, single-compound hazard assessments of BFRs may underestimate the combined ED and cytotoxic hazard of a complex mixture containing BFRs and their metabolites.

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5 References

[1] Darnerud, P. O., Eriksen, G. S., Johanesson, T., Larsen, P. B. *et al.*, Polybrominated diphenyl ethers: ocurrence, dietary exposure, and toxicology, *Environ. Health Perspect.* 2001, *109 (Suppl 1)*, 49 – 68.

[2] Legler, J., Brouwer, A., Are brominated flame retardants endocrine disruptors?, *Environ. Int.* 2003, *29*, 879 – 885.

[3] Vos, J. G., Becher, G., Van den Berg, M., De Boer, J., Leonards, P. E. G., Brominated flame retardants and endocrine disruption, *Pure Appl. Chem.* 2003, *75*, 2039 – 2046.

[4] Hamers, T., Kamstra, J. H., Sonneveld, E., Murk, A. J., *et al.*, In vitro profiling of the endocrine disrupting potency of brominated flame retardants, *Toxicol. Sci.* 2006, *92*, 157 – 173.

[5] Hakk, H., Letcher, R. J., Metabolism in the toxicokinetics and fate of brominated flame retardants – a review, *Environ. Internat.* 2003, *29*, 801 – 828.

[6] Meerts, I. A. T. M., Letcher, R. J., Hoving, S., Marsh, G., *et al.*, In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PBDEs, and polybrominated bisphenol A compounds, *Environ. Health Perspect.* 2001, *109*, 399 – 407.

[7] Örn, U., Klasson-Wehler, E., Metabolism of 2,2′,4,4′-tetrabromodiphenylether in rat and mouse, *Xenobiotica* 1998, *28*, 199 – 211.

[8] Marsh, G., Athanasiadou, M., Athanassiadis, I., Sandholm, A., Identification of hydroxylated metabolites in 2,2′,4,4′-tetrabromodiphenyl ether exposed rats, *Chemosphere* 2006, *63*, 690 – 697.

[9] Malmberg, T., Athanasiadou, M., Marsh, G., Brandt, I., Bergman, Å., Identification of hydroxylated polybrominated diphenyl ether metabolites in blood plasma from polybrominated diphenyl ether exposed rats, *Environ. Sci. Technol.* 2005, *39*, 5342 – 5348.

[10] Bergman, Å., Athanasiadou, M., Fäldt, E., Jakobsson, K., Hydroxylated PBDE metabolites in human blood, *Organohalogen Compd.* 2006, *68*, 635 – 638.

[11] Malmvärn, A., Marsh, G., Kautsy, L., Athanasioadou, M., *et al.*, Hydroxylated and methoxylated brominated diphenyl ethers in the read algae *Ceramium tenuicorne* and blue mussels form the Baltic Sea, *Environ. Sci. Technol.* 2005, *39*, 2990 – 2997.

[12] Meerts, I. A. T. M., Van Zanden, J. J., Luijks, E. A., Van Leeuwen-Bol, I., *et al.*, Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro, *Toxicol. Sci.* 2000, *56*, 95 – 104.

[13] Sanders, J. M., Burka, L. T., Smith, C. S., Black, W., *et al.*, Differential expression of CYP 1A, 2B, and 3A genes in the F344 rat following exposure to a polybrominated diphenyl ether mixture of individual components, *Toxicol. Sci.* 2005, *88*, 127 – 133.

[14] Germer, S., Piersma, A. H., Van der Ven, L., Kamyschnikow, A. *et al.*, Subacute effects of the brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on hepatic cytochrome P450 levels in rats, *Toxicol.* 2006, *218*, 229 – 236.

[15] Harju, M., Hamers, T., Kamstra, J. H., Sonneveld, E., *et al.*, Quantitative structure-activity relationship modelling on in vitro endocrine effects and metabolic stability involving 26 selected brominated flame retardants, *Environ. Toxicol. Chem.* 2007, *26*, 816 – 826.

[16] Örn, U., Eriksson, L., Jakobsson, E., Bergman, Å., Synthesis and characterization of 32 polybrominated diphenyl ethers – unlabelled and radiolabelled tetra-, penta- and hexa-bromodiphenyl ethers, *Acta Chem. Scand.* 1996, *50*, 802 – 807.

[17] Marsh, G., Hu, J., Jakobsson, E., Rahm, S., Bergman, Å., Synthesis and characterization of 32 polybrominated diphenyl ethers, *Environ. Sci. Technol.* 1999, *33*, 3033 – 3037.

[18] Marsh, G., Stenutz, R., Bergman, Å., Synthesis of hydroxylated and methoxylated polybrominated diphenyl ethers – natural products and potential polybrominated diphenyl ether metabolites, *Eur. J. Org. Chem.* 2003, *14*, 2566 – 2576.

[19] Hovander, L., Athanasiadou, M., Asplund, L., Jensen, S., Klasson-Wehler, E., Extraction and cleanup methods for analysis of phenolic and neutral organohalogens in plasma, *J. Anal. Toxicol.* 2000, *24*, 696 – 703.

[20] Van Lipzig, M. M. H., Commandeur, J. N., De Kanter, F. J. J., Damsten, M. C., *et al.*, Bioactivation of dibrominated biphenyls by cytochrome P450 activity to metabolites with estrogenic activity and estrogen sulfotransferase inhibition capacity, *Chem. Res. Toxicol.* 2005, *18*, 1691 – 1700.

[21] Lans, M. C., Klasson-Wehler, E., Willemsen, M., Meussen, E., *et al.*, Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-*p*-dioxines and -dibenzofurans with human transthyretin, *Chem. Biol. Interact.* 1993, *88*, 7 – 21.

[22] Kester, M. H. A., Bulduk, S., Tibboel, D., Meinl, W., *et al.*, Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs, *Endocrinology* 2000, *141*, 1897 – 1900.

[23] Sonneveld, E., Jansen, H. J., Riteco, J. A. C., Brouwer, A., Van der Burg, B., Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays, *Toxicol. Sci.* 2005, *83*, 136 – 148.

[24] Schreurs, R. H., Sonneveld, E., Jansen, J. H., Seinen, W., Van der Burg, B., Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR) and progesterone receptor (PR) in reporter gene bioassays, *Toxicol. Sci.* 2005, *83*, 264 – 272.

[25] Legler, J., Van den Brink, C. E., Brouwer, A., Murk, A. J., *et al.*, Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line, *Toxicol. Sci.* 1999, *48*, 55 – 66.

[26] Murk, A. J., Legler, J., Denison, M. S., Giesy, J. P., *et al.*, Chemical-activated luciferase gene expression (CALUX): a novel in vitro bioassay for Ah receptor active compounds in sediments and pore water, *Fund. Appl. Toxicol.* 1996, *33*, 149 – 160.

[27] Gutleb, A. C., Meerts, I. A. T. M., Bergsma, J. H., Schriks, M., Murk, A. J., T-Screen as a tool to identify thyroid hormone receptor active compounds, *Environ. Toxicol. Pharm.* 2005, *19*, 231 – 238.

[28] O'Brien, J., Wilson, I., Pognan, F., Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity, *Eur. J. Biochem.* 2000, *267*, 5421 – 5426.

[29] Van Boxtel, A. L., Kamstra, J. H., Cenijn, P. H., Pieterse, B., *et al.*, A phenolic PBDE (6-OH-BDE47) is acutely toxic in zebrafish by uncoupling of oxidative phosphorylation. *Environ. Sci. Technol.* (in press).

[30] Kamstra, J. H., Du, T., Visser, T. J., Van der Ven, L. T. M., *et al.*, In vitro induction of organic anion transporting polypeptides (OATPs) as a possible mechanism for thyroid hormone disruption by hexabromocyclododecane (HBCD), *BFR2007 Amsterdam – 4th International Workshop on Brominated Flame Retardants*, Amsterdam, The Netherlands, 24 – 27 April 2007, http://www.BFR2007.com.

[31] Kierkegaard, A., Burreau, S., Marsh, G., Klasson-Wehler, E., *et al.*, Metabolism and distribution of 2,2′,4,4′-tetrabromo[^{14}C]diphenyl ether in pike (Esox lucius) after dietary exposure, *Organohalogen Compd.* 2001, *52*, 58 – 61.

[32] COMPARE. *Comparison of exposure-effect pathways to improve the assessment of human health risks of complex environmental mixtures of organohalogens – Integrated and comparative risk assessment for exposure to complex mixtures of environmental chemicals*, 2005, http://ec.europa.eu/research/endocrine/pdf/compare_risk_assessment_document _en.pdf.

[33] Cuadra, S. N., Linderholm, L., Athanasiadou, M., Jakobsson, K., Persistent organochlorine pollutants in children working at a waste-disposal site and in young females with high fish consumption in Managua, Nicaragua, *Ambio* 2006, *35*, 109 – 116.

